

## Cell Anchorage Permits Efficient Signal Transduction Between Ras and Its Downstream Kinases\*

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**Cell anchorage strongly affects the signal transduction cascade initiated by peptide mitogens. For both epidermal growth factor and platelet-derived growth factor, activation of the consensus mitogen-activated protein kinase cascade is impaired when cells are held in suspension as compared with cells anchored to a fibronectin substratum. Upstream events in the signaling cascade, including tyrosine phosphorylation of the mitogen receptor and GTP loading of Ras, are similar in anchored and suspended cells. However, propagation of the signal to Raf and subsequently to the downstream kinases MEK and mitogen-activated protein kinase is markedly attenuated in suspended cells. Thus, there seems to be a distinct anchorage-dependent step between Ras and Raf in the signaling cascade initiated by peptide mitogens. These observations may have important implications for understanding the anchorage dependence of cell growth.**

Cell anchorage to the proteins of the extracellular matrix is known to have profound effects on cell differentiation (1, 2), cell growth (3), and apoptosis (4). A particularly important example of this concerns the recently described effects of anchorage on the expression and activity of components of the cell cycle machinery, including cyclin D1-CDK4,6 complexes and cyclin E-CDK2 complexes (5, 6). These observations are clearly relevant to the question of why both soluble mitogens and cell anchorage are required for the growth of normal cells, whereas the anchorage requirement is abrogated in transformed cells (7). Many aspects of cell to extracellular matrix interactions involve the integrin family of cell surface heterodimeric adhesion proteins (8). Recently, it has become clear that integrins are signal transducing receptors (9, 10) capable of influencing a number of intracellular biochemical activities including protein tyrosine kinases (11), serine/threonine kinases (12), and ionic transients (13). In particular, integrin-mediated cell adhesion

can trigger activation of MAP<sup>1</sup> kinase (14–16) and of other protein kinases (17) that are part of the consensus signaling pathway leading from receptor tyrosine kinases to Ras and then to a cytoplasmic kinase cascade comprising Raf, MEK1, MEK2, and MAP kinases (18).

Because integrins directly activate elements of the MAP kinase cascade, it is of interest to ask whether integrin-mediated cell anchorage can also regulate the action of soluble mitogens on this cascade. If this were so, it would have important ramifications for understanding the anchorage dependence of cell cycle traverse. Previous studies of possible collaboration between peptide mitogens and cell anchorage have led to differing results. In some cases an enhancement of mitogen signaling was observed in anchored cells as compared with their counterparts maintained in suspension, whereas in other cases no such effect was observed (19–22). In the present investigation we have studied the collaboration between mitogens and anchorage in NIH 3T3 cells, a cell type that has been widely used in signal transduction studies. We have examined several steps in the signal transduction pathway leading from receptor tyrosine kinases to Ras and then to the downstream kinases. We find that peptide mitogen activation of receptor tyrosine kinases and subsequent activation of Ras are independent of anchorage. However, signal transduction between Ras and Raf is markedly attenuated in nonadherent cells, leading to reduced activation of Raf, MEK, and MAP kinase.

### EXPERIMENTAL PROCEDURES

NIH 3T3 cells were maintained in Dulbecco's minimal essential medium containing 10% bovine calf serum and antibiotics. Confluent cells were serum-starved for 16 h before detachment by 0.05% trypsin and 0.33 mM EDTA; trypsin activity was neutralized by 1 mg/ml soybean trypsin inhibitor. Cells were suspended in Dulbecco's minimal essential medium with 2% bovine serum albumin and incubated in suspension at 37 °C for 45 min in a rotator to allow kinases become quiescent. Cells were then either maintained in suspension or plated onto dishes coated with fibronectin (20 µg/ml) or with poly-L-lysine (20 µg/ml) and incubated at 37 °C for the indicated times. In some cases the suspended or adherent cells were stimulated with either EGF or PDGF (Upstate Biotechnologies Inc.). Cell lysates were prepared and tested for the activity of Raf, MEK, and MAP kinase using specific *in vitro* kinase assays as described previously (17). The phosphorylation status of the EGF receptor and PDGF receptor were evaluated by immunoprecipitation of the receptor using antibodies obtained from H. S. Earp (EGF-R) or from Santa Cruz Biotechnology (PDGF-R) followed by Western blotting with an anti-phosphotyrosine antibody and detection by enhanced chemiluminescence (17). For studies of GTP loading of the Ras protein, cells were radiolabeled with [<sup>32</sup>P]orthophosphate, and the [<sup>32</sup>P]GTP and GDP bound to immunoprecipitated Ras were quantitated by thin layer chromatography and PhosphorImager analysis as described (17).

### RESULTS AND DISCUSSION

Because elements of the MAP kinase cascade are directly but transiently activated by integrin-mediated cell adhesion (17), we initially examined the kinetics of this process to find a time point when we could examine anchorage effects on mitogen-driven activation of MAP kinase without a direct contribution from integrin-mediated MAP kinase activation. As seen in Fig. 1 (A and B), when NIH 3T3 cells were held in suspension, EGF caused a robust tyrosine phosphorylation of EGF-R but had

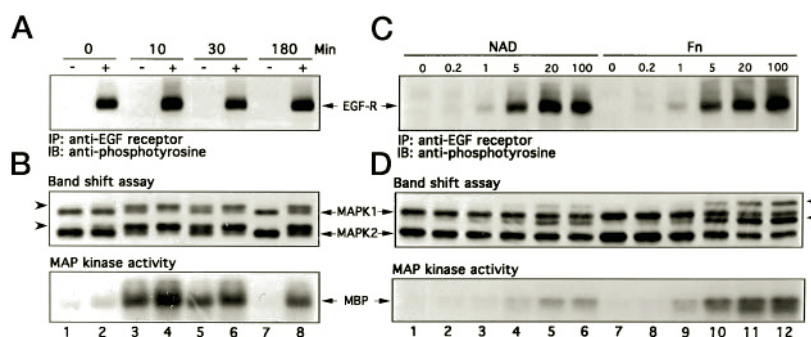
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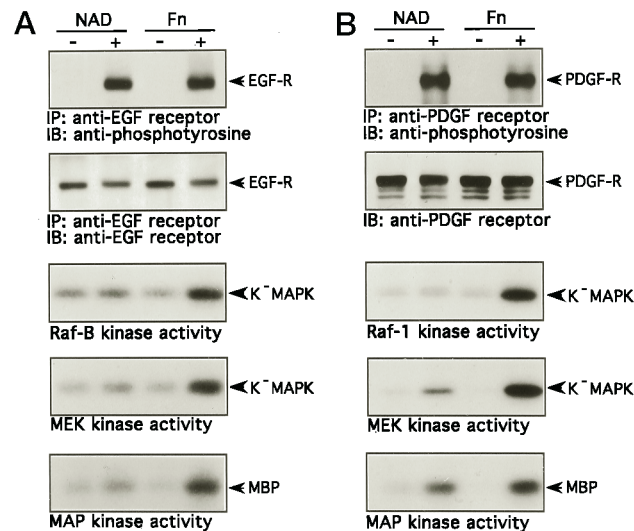
<sup>1</sup> The abbreviations used are: MAP, mitogen-activated protein; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; EGF-R, EGF receptor; PDGF-R, PDGF receptor.



**FIG. 1. Cell anchorage to fibronectin modulates MAP kinase activation but not EGF receptor activation.** Serum-starved NIH 3T3 cells were harvested and then either maintained in suspension or allowed to adhere to substrata coated with fibronectin; in some cases (+) the cells were treated with EGF for 5 min. Tyrosine phosphorylation of the EGF receptor was evaluated by immunoprecipitation (IP) with anti-EGF-R antibody followed by Western immunoblotting (IB) with an anti-phosphotyrosine antibody. MAP kinase activity was evaluated by a band shift assay and by immunoprecipitation of the enzyme followed by an *in vitro* kinase assay using myelin basic protein (MBP) as a substrate as described previously (17). **A**, EGF-R tyrosine phosphorylation as a function of time after plating cells on fibronectin. **B**, MAP kinase activity as a function of time after plating cells on fibronectin. (For **A** and **B** 20 ng/ml of EGF was used.) **C**, EGF-R tyrosine phosphorylation as a function of the dose of EGF. **D**, MAP kinase activation as a function of the dose of EGF. (For **C** and **D** the EGF dose is given in ng/ml.) **NAD**, nonadherent (suspended) cells; **FN**, cells adherent to fibronectin-coated substrata.

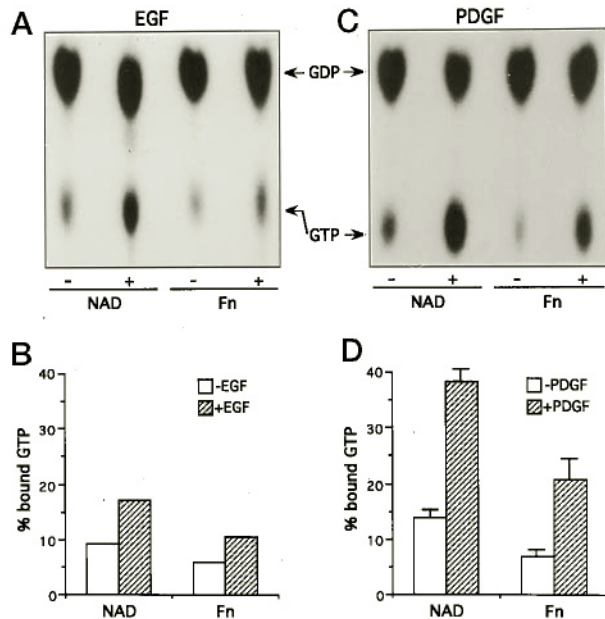
only a very modest effect on MAP kinase (Fig. 1*B*, lanes 1 and 2). After 10 min of cell adhesion to fibronectin-coated substrata, when the cells were fully attached but not spread, there was a strong adhesion-mediated activation of MAP kinase; EGF stimulation caused tyrosine phosphorylation of EGF-R and further stimulated MAP kinase (Fig. 1*B*, lanes 3 and 4). A qualitatively similar situation also prevailed after 30 min of cell adhesion when the cells were partially spread (Fig. 1*B*, lanes 5 and 6). By 180 min, when the cells were well spread, in the absence of EGF there was only a basal level of MAP kinase activity, whereas treatment with EGF caused tyrosine phosphorylation of EGF-R and resulted in a strong stimulation of MAP kinase (Fig. 1*B*, lanes 7 and 8). Thus, in serum-starved 3T3 cells, EGF activation of its receptor seems to be independent of cell anchorage; however, the MAP kinase response is strongly influenced by anchorage. In nonadherent cells, EGF produces only a weak activation of MAP kinase. Shortly after the cells adhere to the fibronectin substratum, EGF and anchorage have approximately additive effects on MAP kinase activity. At longer times, EGF strongly activates MAP kinase in anchored cells, whereas the direct activation by cell adhesion has returned to basal levels. In Fig. 1 (*C* and *D*) we examined EGF concentration-response relationships for EGF-R and MAP kinase in cells that have either been maintained in suspension or anchored to fibronectin for 180 min. As shown, the concentration-response profile for EGF-R tyrosine phosphorylation was essentially identical in suspended cells and cells anchored to fibronectin substrata. However, at all EGF concentrations tested, anchored cells displayed a 3–4-fold greater activation of MAP kinase than did suspended cells (for example, compare lanes 4 and 10 in Fig. 1*D*, both at 5 ng/ml EGF).

We have also investigated how anchorage modulates mitogenic actions on other components of the MAP kinase cascade. As shown in Fig. 2*A*, EGF stimulated similar levels of tyrosine phosphorylation of EGF-R in suspended or anchored cells. However, EGF produced substantially stronger activations of Raf-B, MEK, and MAP kinase in cells anchored to fibronectin substrata as compared with nonanchored cells. Raf-1 was also activated (weakly) by EGF in anchored cells but not in suspended cells (not shown). We decided to also examine anchorage dependence of signaling events mediated by PDGF, another peptide mitogen. Exposure of 3T3 cells to PDGF caused a substantially greater increase in overall cellular protein tyrosine phosphorylation than was observed with EGF (not shown). As seen in Fig. 2*B*, PDGF caused equivalent robust tyrosine phosphorylation of its cognate receptor in both anchored and



**FIG. 2. Mitogen activation of the MAP kinase cascade in anchored or suspended cells.** Serum-starved NIH 3T3 cells were harvested and then either maintained in suspension or allowed to adhere to substrata coated with fibronectin for 180 min; in some cases (+) the cells were treated with either EGF (20 ng/ml) or PDGF (20 ng/ml) for 5 min. The components of the signaling cascade were immunoprecipitated (IP) using specific antibodies, and their activities were assayed as described previously (17). **A**, EGF responses. *Top panel*, EGF-R, Western blot (IB) with anti-phosphotyrosine antibody; *second panel*, EGF-R expression, Western blot with anti-EGF-R; *third panel*, Raf-B activation, immunoprecipitation with anti-Raf-B followed by an *in vitro* linked kinase assay (17) with kinase-dead MAP kinase ( $K^-$  MAPK) as a substrate; *fourth panel*, MEK activation, immunoprecipitation with anti-MEK followed by an *in vitro* kinase using kinase-dead MAP kinase as a substrate (17); *fifth panel*, MAP kinase activation, immunoprecipitation with anti-MAP kinase followed by an *in vitro* kinase assay using MBP as a substrate (17). **B**, PDGF responses. The same assays were used as in **A**, except that the *top two panels* employed specific antibodies to PDGF-R (Santa Cruz), and the *third panel* employed an antibody to Raf-1. **NAD**, nonadherent cells; **FN**, cells adherent to fibronectin-coated substrata; **MBP**, myelin basic protein.

suspended cells. However, PDGF treatment resulted in markedly stronger activation of Raf-1 and MEK in anchored cells as compared with suspended cells, as well as a more modest but significant difference in MAP kinase activation. Thus, for PDGF, as for EGF, cell anchorage seems to control the efficiency of signal transduction between initial activation of the receptor tyrosine kinase and subsequent activation of downstream kinases.

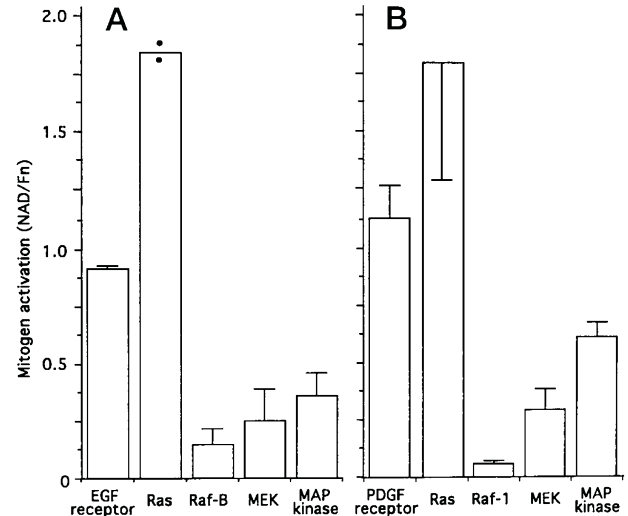


**FIG. 3. Mitogen activation of Ras GTP loading in anchored or suspended cells.** Serum-starved NIH 3T3 cells were harvested and then either maintained in suspension or allowed to adhere to substrata coated with fibronectin for 180 min; in some cases (+) the cells were treated with EGF (20 ng/ml) or PDGF (20 ng/ml) for 5 min. Ras GTP loading was measured by thin layer chromatography as described (17). The data are presented as the percentage of bound GTP ( $(\text{GTP}/(\text{GTP} + 1.5 \text{ GDP} + \text{GTP})) \times 100$ ). The results for PDGF represent the means and standard errors for three independent experiments, whereas for EGF the means of two independent experiments are shown.

Because the GTP-bound form of Ras is a key transducer in the mitogen signaling pathway (18), we decided to examine Ras GTP loading in anchored or suspended 3T3 cells treated with either PDGF or EGF. As seen in Fig. 3, the basal GTP/GDP ratio was somewhat higher in suspension cells than in adherent cells. Treatment with peptide mitogen resulted in a strong increase in Ras GTP loading in both suspended cells and anchored cells, with PDGF producing a somewhat greater effect than EGF. Thus, Ras GTP loading in response to mitogens occurred in suspended cells at least as well as in anchored cells; in fact, suspended cells usually showed higher levels of GTP loading than anchored cells. This observation, along with those of Fig. 2, suggests that peptide mitogen signaling pathways are intact and operate efficiently in both suspended and anchored cells up to the level of Ras but are attenuated between Ras and the Raf kinases in the nonanchored cells.

Quantitation of anchorage effects on activation of several components of the EGF- and PDGF-triggered pathways is shown in Fig. 4. The data are expressed as the ratio of the mitogen activation in suspended cells *versus* anchored cells. The downstream kinases (Rafs, MEKs, and MAP kinases) display substantial reductions in activity in nonanchored cells, but Ras and the receptor tyrosine kinases do not. Although the exact magnitudes of the anchorage effects on activation of the individual components of the pathway differ somewhat between EGF and PDGF, the trend is similar. The observation that EGF and PDGF produce qualitatively similar but quantitatively distinct effects on the consensus MAP kinase cascade is not surprising, because individual receptor tyrosine kinases are known to have distinct effects on cell growth and differentiation (23).

Although cell anchorage to a fibronectin-coated substratum clearly has a substantial impact on the MAP kinase cascade, it is not yet certain that this is purely an integrin-mediated phenomenon. Preliminary experiments have shown that EGF



**FIG. 4. Attenuation of the mitogen signaling cascade between Ras and Raf in nonanchored cells.** This figure summarizes several independent experiments for EGF (A) and PDGF (B) stimulation. The tyrosine phosphorylation of immunoprecipitated EGF-R and PDGF-R was quantitated by laser densitometry of enhanced chemiluminescence Western blots. The activation of Raf, MEK, and MAP kinases, as well as GTP loading of Ras, were quantitated using a PhosphorImager. The parameter shown on the ordinate (Mitogen Activation NAD/Fn) represents the ratio of mitogen activation in nonadherent cells (NAD) to that observed in cells anchored on fibronectin substrata (Fn). In each case it was calculated by subtracting the basal value from the mitogen-stimulated value; the differences (termed  $\delta\text{NAD}$  or  $\delta\text{Fn}$ ) were divided to get the ratio given on the ordinate ( $\delta\text{NAD}/\delta\text{Fn}$ ). Thus, if cell anchorage had no effect on the mitogen activation of a certain component in the pathway, the ratio on the ordinate for that component would be 1; numbers less than 1 indicate that the mitogen-mediated activation is attenuated in nonadherent cells. The results represent the means and standard errors for three independent experiments (two for EGF effects on Ras).

activation of MAP kinase is 2–3-fold greater in cells plated on a fibronectin substratum, as opposed to a poly-lysine substratum (data not shown). This suggests that the anchorage effects on signaling that we have observed may be mediated by integrins; however, several additional lines of investigation will be needed to fully confirm this possibility.

Anchorage dependence of cell growth is one of the most fundamental differences between normal and transformed cells (24). Our observations indicate that cell anchorage can influence the efficiency of signal transduction in mitogenic pathways. This suggests the possibility that adhesion effects on early signaling events may play an important role in anchorage dependence of cell growth, although other factors may also be involved. For both EGF and PDGF, the upstream events of the mitogen signaling pathway were independent of anchorage. Thus, receptor tyrosine kinase activation and GTP loading of Ras were robust in both anchored and suspended cells. For both mitogens, however, cell adhesion had a profound effect on the activation of the MEK-kinases Raf-B and Raf-1 and clear-cut effects of lesser magnitude on MEK and MAP kinase. The nonlinearity of the effects we have observed on anchorage regulation of signal transduction may be due to the extensive branching and cross-talk that is known to occur in the MAP kinase cascade (25). Our observations suggest that in suspended cells, there is a rather sharp break in the signaling cascade between Ras and the Raf kinases. Because a major role of Ras in signal transduction is to recruit Raf to the plasma membrane (26), our findings suggest that cell anchorage contributes to this process. One plausible model is that integrin-dependent focal contacts formed during cell adhesion participate in the recruitment and subsequent activation of Raf.



Cell anchorage to fibronectin, a process primarily mediated by integrins, resulted in 2–3-fold greater activation of MAP kinases by peptide mitogens as compared with suspended cells. At this point it is unclear whether a change in MAP kinase activation of this magnitude would account for the strong effect that anchorage has on cell growth. It is important to note, however, that the effects of anchorage on Raf-1 or Raf-B activation were much greater (8–20-fold). Raf family kinases are thought to have downstream targets other than the MAP kinase pathway (27, 28). Thus, it seems possible that the anchorage modulation of mitogen signaling reported here, particularly the dramatic effect on Raf family kinases, may be important aspects of cell growth control.

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